

Letter to the Editor

Oroscomucoid like protein 3 (ORMDL3) transgenic mice have reduced levels of sphingolipids including sphingosine-1-phosphate and ceramide

To the Editor:

Oroscomucoid like protein 3 (ORMDL3) is a gene localized to chromosome 17q21, which has been highly linked to asthma in genomewide association studies.¹ The single nucleotide polymorphism (SNP) that links ORMDL3 to chromosome 17q21 is associated with increased levels of ORMDL3 expression.² *In vitro* studies have demonstrated that ORMDL3 inhibits sphingolipid synthesis by forming a complex with the enzyme serine palmitoyl transferase (SPT), the first and rate-limiting enzyme in sphingolipid synthesis³ (see Fig E1 in this article's Online Repository at www.jacionline.org). At present, it is not known whether increased expression of ORMDL3 *in vivo* (as might occur in patients with asthma having the SNP linked to chromosome 17q21 and increased ORMDL3 expression) is associated with inhibition of sphingolipid synthesis. In this study, we have measured levels of sphingolipids *in vivo* in mice we have previously generated to express increased

levels of human ORMDL3⁴ to determine whether they have reduced levels of sphingolipids as hypothesized on the basis of previous *in vitro* studies of ORMDL3 and sphingolipids.³ We have previously demonstrated that human ORMDL3 Zona Pellucida 3 Cre (hORMDL3^{zp3-Cre}) mice that overexpress human ORMDL3 universally⁴ exhibit an asthma phenotype characterized by increased airway responsiveness (AHR) and increased airway remodeling (increased peribronchial smooth muscle, increased peribronchial fibrosis) when these mice have no evidence of increased airway inflammation.⁴

We measured levels of sphingolipids including sphinganine, ceramide, sphingosine, and sphingosine 1 phosphate (S1P) in serum and lung obtained from hORMDL3^{zp3-Cre} mice or from wild-type (WT) littermate control mice (n = 4/group) using liquid chromatography-tandem mass spectrometry⁵ (see this article's Methods section in the Online Repository at www.jacionline.org). Our studies demonstrated that hORMDL3^{zp3-Cre} mice had significantly reduced serum levels of sphingolipids including sphinganine ($P < .02$), ceramide 24:0 ($P < .01$), sphingosine ($P < .02$), and S1P ($P < .02$) compared with WT control (Fig 1). Levels of only serum ceramide 24:0 (but not ceramide 18:0, ceramide 20:0, ceramide 22:0, and ceramide 24:1) were decreased in serum in hORMDL3^{zp3-Cre} mice (see Fig E2 in

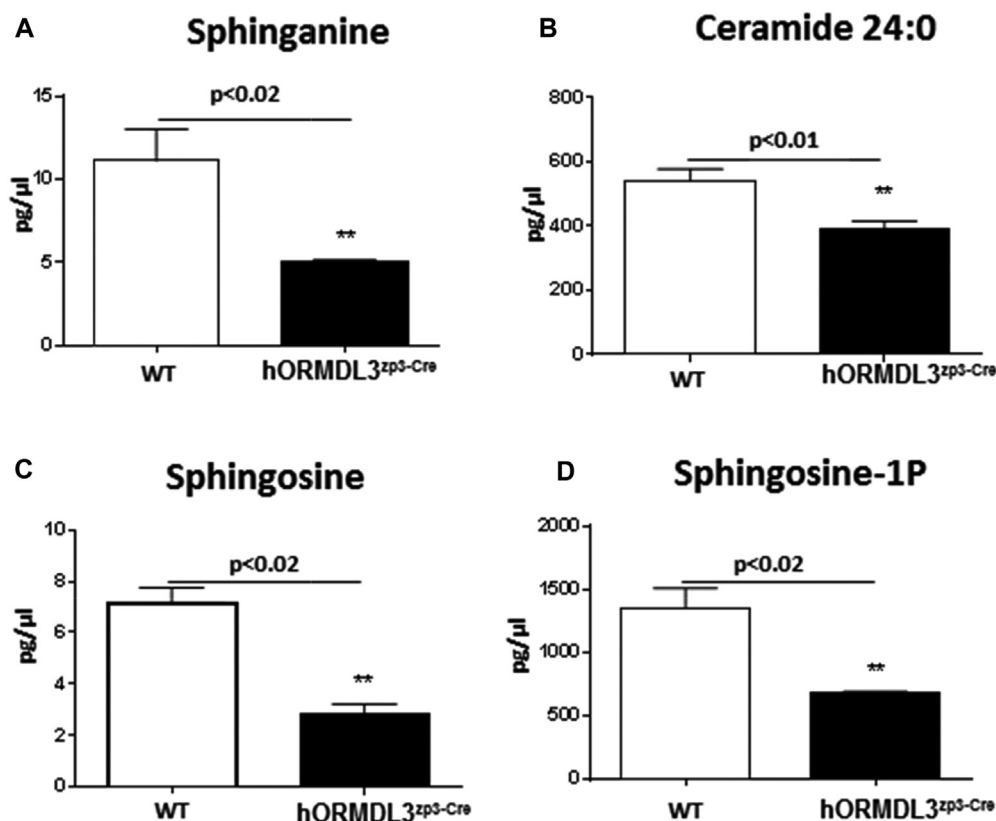


FIG 1. Levels of serum sphingolipids in hORMDL3^{zp3-Cre} mice. Levels of serum sphingolipids in hORMDL3^{zp3-Cre} mice and WT mice were quantitated by LC-MS/MS. hORMDL3^{zp3-Cre} mice had significantly reduced serum levels of sphingolipids including (A) sphinganine ($P < .02$), (B) ceramide 24:0 ($P < .01$), (C) sphingosine ($P < .02$), and (D) S1P ($P < .02$) compared with WT control. LC-MS/MS, Liquid chromatography-tandem mass spectrometry. Fig 1, A, C, and D: ** $P < .02$. Fig 1, B: ** $P < .01$.

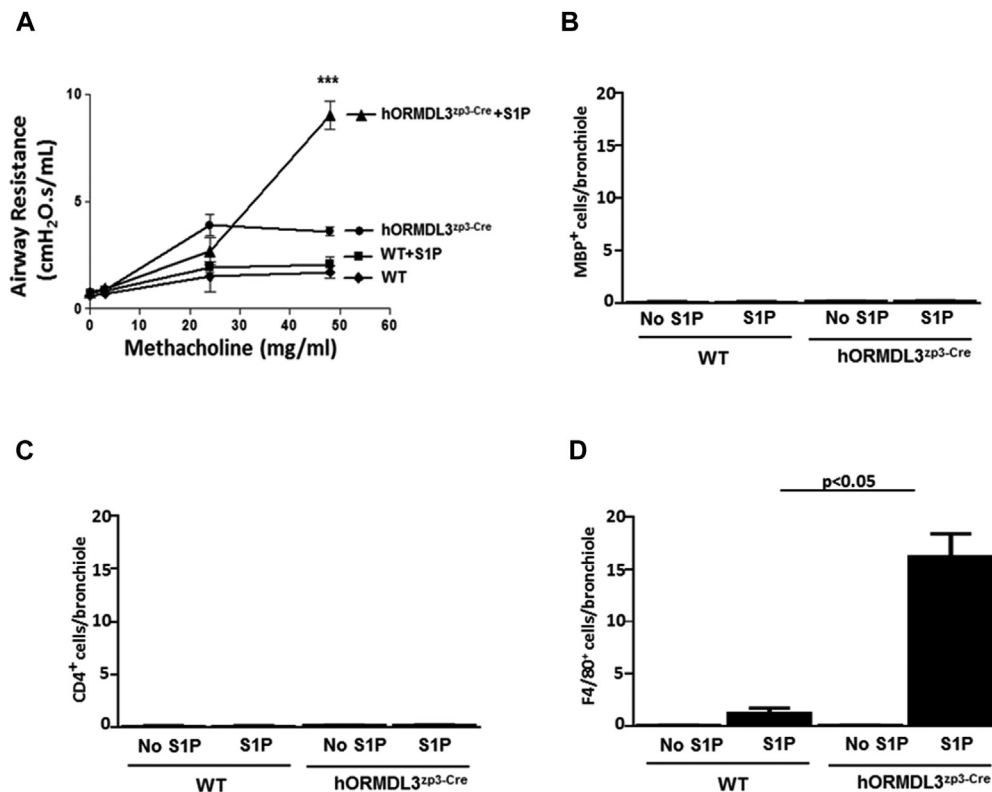


FIG 2. S1P increases AHR in hORMDL3^{zp3-Cre} mice. **A**, Administration of intranasal S1P, 10 ng on day 0, and 10 ng on day 7, to hORMDL3^{zp3-Cre} mice significantly increases AHR compared with hORMDL3^{zp3-Cre} mice not administered S1P ($P < .05$), or compared with WT mice administered S1P ($P < .05$). No allergen was administered to any group of mice. Levels of peribronchial MBP⁺ eosinophils (**B**), CD4⁺ cells (**C**), and F4/80⁺ macrophages (**D**) were quantitated by immunohistochemistry in lung sections. MBP, Major basic protein. *** $P < .05$.

this article's Online Repository at www.jacionline.org). Thus, our study provides novel evidence that ORMDL3 does inhibit the synthesis of sphingolipids downstream of the enzyme SPT in serum *in vivo*. We also measured lung extract levels of sphingolipids and noted a modest reduction in sphinganine levels in hORMDL3^{zp3-Cre} mice ($P < .05$ vs WT control), but no difference in lung levels of ceramide 24:0, sphingosine, or S1P (see Fig E3 in this article's Online Repository at www.jacionline.org). Previous studies have demonstrated that mice heterozygous for SPT (Sptlc2^{+/-}), or mice administered myriocin, an inhibitor of SPT, had decreased synthesis of selected lung sphingolipid synthesis (sphinganine and ceramide) with no change in levels of lung sphingosine or S1P.⁶ Heterozygous Sptlc2^{+/-} mice have approximately 60% decreased hepatic SPT activity, decreased hepatic sphinganine, ceramides, and sphingosine, and decreased serum ceramides and sphingosine levels but no other apparent phenotype.^{E1} Thus, this study of hORMDL3^{zp3-Cre} mice and previous studies of Sptlc2^{+/-} mice both demonstrate that in the lung there is reduction in sphinganine levels (and ceramide in the case of Sptlc2^{+/-} mice), but no reduction in end products of this pathway, namely, sphingosine and S1P in both strains of mice. At present it is unknown why hORMDL3^{zp3-Cre} mice inhibits sphingolipid synthesis in serum from the first step to the last step of the pathway regulated by SPT, but inhibit only the proximal step of the pathway in lung. Potential explanations that will need to be explored include the

possibility that ORMDL3 may influence the generation of sphingolipid synthesis differently in different cell types (we have studied universal increased expression of ORMDL3), that the breakdown of sphingolipids is different in lung versus serum, or that a sphingolipid salvage pathway contributes to the differences noted. In addition, studies have shown that a complex stoichiometric interaction between SPT and ORMDL3 may have a bearing on sphingolipid levels, rather than simple expression levels.^{E2} Studies have also demonstrated that *in vitro* small increases in ORMDL3 expression in lung epithelial cells and macrophages decreased ceramide levels, whereas higher expression of ORMDL3 increased ceramide production, suggesting that ORMDL3 regulates ceramide levels in a complex manner.^{E3}

Because increased ORMDL3 reduces S1P, we performed additional experiments in which we administered S1P intranasally to naive WT mice as well as to naive hORMDL3^{zp3-Cre} mice (see this article's Methods section in the Online Repository). Interestingly, the hORMDL3^{zp3-Cre} mice administered S1P had a significant further increase in AHR compared with WT mice administered S1P, as well as compared with hORMDL3^{zp3-Cre} mice not administered S1P (Fig 2). This increase in AHR in hORMDL3^{zp3-Cre} mice was not associated with increased peribronchial eosinophils or CD4 lymphocytes, but was associated with increased peribronchial macrophages (Fig 2). S1P has previously been shown to be involved in macrophage trafficking^{E4} and

a chemoattractant for macrophages.^{E5} At present, we do not know the mechanism of this increased AHR and macrophage response, which will require further study. However, the increased AHR response to S1P when administered to the airway of naive hORMDL3^{zp3-Cre} mice provides evidence that increasing levels of S1P in the lungs of mice that have reduced levels of S1P contribute to increased AHR.

There are several studies of sphingolipids that suggest that increased levels of S1P (the final product in the sphingolipid pathway) are likely to contribute to AHR in asthma. For example, administration of S1P to WT mice significantly increases AHR,⁷ whereas inhalation of an Sphk1 inhibitor inhibits asthma outcomes in mouse models.^{8,E6} In addition, S1P is known to induce smooth muscle contraction,^{E7} as well as influence activation and function of mast cells, eosinophils, and dendritic cells.⁷ The importance of S1P to asthma is suggested from studies demonstrating increased bronchoalveolar lavage levels of S1P in human patients with allergic asthma following endobronchial allergen challenge,^{E7} and genetic linkage studies showing an association between functional SNPs in the S1P receptor-1 and asthma.^{E8} Thus, whether increased expression of ORMDL3 in hORMDL3^{zp3-Cre} mice increases AHR in the absence of airway inflammation through reductions in sphingolipid levels is supported by some,⁶ but not all, studies of sphingolipids.^{7,8,E6,E7}

Interestingly, in Sptlc2^{+/-} mice, the reduced synthesis of sphinganine and ceramide was associated with increases in AHR in the absence of inflammation,⁶ a phenotype we have observed in hORMDL3^{zp3-Cre} mice.⁴ However, there are differences in the profile of sphingolipids reduced in hORMDL3^{zp3-Cre} mice⁴ (sphinganine, sphingosine, S1P, ceramide in serum; sphinganine in lung) compared with Sptlc2^{+/-} mice (sphinganine, ceramide in lung). In addition, hORMDL3^{zp3-Cre} mice⁴ develop spontaneous airway remodeling and mucus expression not observed in Sptlc2^{+/-} mice, suggesting that pathways to remodeling and mucus are independent of the ability of ORMDL3 to inhibit SPT and sphingolipid synthesis.

Studies have also examined the role of ceramide in mouse asthma models and demonstrated that allergen challenge increased lung levels of ceramide in WT mice,^{E3} whereas pretreatment with pharmacologic inhibitors of ceramide reduced AHR, lung eosinophils, and T_H2 cytokines.^{E3} These studies suggest that the reductions in serum ceramide levels we have noted in hORMDL3^{zp3-Cre} mice should be associated with reduced AHR, rather than the increased AHR we have noted in hORMDL3^{zp3-Cre} mice. Thus, the reduced serum ceramide levels in ORMDL3 TG mice, as well as the reduced lung ceramide levels in Sptlc2^{+/-} mice, do not adequately explain the AHR changes we have noted in these mice.

In summary, in this study we have demonstrated that hORMDL3^{zp3-Cre} mice had significantly reduced serum levels of the pathway of sphingolipids regulated by SPT (sphinganine, ceramide, sphingosine, and S1P), as well as reduced lung levels of sphinganine. In addition, we demonstrate that administration of S1P to naive hORMDL3^{zp3-Cre} mice further increases their AHR associated with increased levels of peribronchial macrophages. These *in vivo* studies extend previous *in vitro*

observations that ORMDL3 inhibits the generation of sphingolipids including ceramide and S1P.³ Previous studies have also demonstrated that increased ORMDL3 activates the ATF6 α pathway of the endoplasmic reticulum unfolded protein response and that this regulates levels of SERC2b, which can contribute to AHR.^{4,9} In addition, ORMDL3 regulates levels of remodeling genes (TGF- β 1, ADAM8) as well as CC and CXC chemokines implicated in asthma.⁹ Further studies are needed to determine which ORMDL3-regulated pathway (eg, inhibition of sphingolipid synthesis, inhibition of ATF6 α and SERCA2b, inhibition of remodeling genes, or other as yet unidentified pathways)^{E9,E10} could contribute to increased AHR observed in hORMDL3^{zp3-Cre} mice, and whether in patients with asthma SNPs associated with increased ORMDL3 expression result in reduced sphingolipid levels. Such insights are important in understanding why ORMDL3 on chromosome 17q21 is highly linked to human asthma.

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METHODS

Measurement of sphingolipids using liquid chromatography-tandem mass spectrometry

We measured levels of sphingolipids including sphinganine, ceramide, sphingosine, and S1P in serum and lungs obtained from hORMDL3^{zp3-Cre} mice that overexpress human ORM DL3 universally,^{E11} or from littermate control mice (n = 4/group; 2 males and 2 females; age 12 weeks; all on a C57Bl6 background^{E11}) using liquid chromatography-tandem mass spectrometry methodology at the University of Texas South Western Metabolic Phenotyping Core.^{E12} Briefly, lung samples were homogenized and extracted in a known volume of organic solvent, while serum samples in cryovials were thawed in a water bath at 37°C for 10 minutes, and then in an ice bath for aliquoting. Fifty microliters of serum was added to 2 mL of organic extraction solvent (isopropanol: ethyl acetate, 15:65; v:v) in a borosilicate glass screw cap tube. Immediately afterward, 20 µL of internal standard solution was added (Avanti Polar Lipids, AL Ceramide/Sphingoid Internal Standard Mixture II diluted 1:10 in ethanol). The mixture was vortexed and sonicated in an ultrasonic bath for 10 minutes at 40°C. The samples were then allowed to reach room temperature, and 2-phase liquid extraction was performed. The supernatant was transferred to a new tube, and the protein precipitate was re-extracted. Supernatants were combined and evaporated under nitrogen. The dried residue was reconstituted in 200 µL of HPLC solvent B (methanol/formic acid 99:1; vol:vol containing 5 mM ammonium formate) for liquid chromatography-tandem mass spectrometry analysis using a Shimadzu Nexera X2 UHPLC system coupled to a Shimadzu LCMS-8050 triple quadrupole mass spectrometer. Quantitative analysis of sphingolipids was achieved using selective reaction-monitoring scan mode.

Administration of S1P to hORMDL3^{zp3-Cre} mice and measurement of AHR and lung inflammation

Two intranasal doses (10 ng each) of S1P, 1 dose on day 0, and 1 dose on day 7, were administered to naive 12-week-old hORMDL3^{zp3-Cre} mice or littermate WT mice all on a C57Bl6 background⁴ who were sacrificed on day 21. Naive 12-week-old hORMDL3^{zp3-Cre} mice or littermate WT mice not administered S1P served as controls. No allergen was administered to any group of mice (n = 8/group). The dose of S1P chosen is the same dose used in previous studies of subcutaneous S1P in mouse models of asthma.^{E13} AHR to methacholine was assessed in intubated and ventilated hORMDL3^{zp3-Cre} mice and WT mice using flexiVent as previously described in this laboratory.^{E14} CD4⁺ T cells, major basic protein⁺ eosinophils, and F4/80⁺ macrophages

were quantitated in peribronchial regions of lungs by immunohistochemistry as previously described.^{E14}

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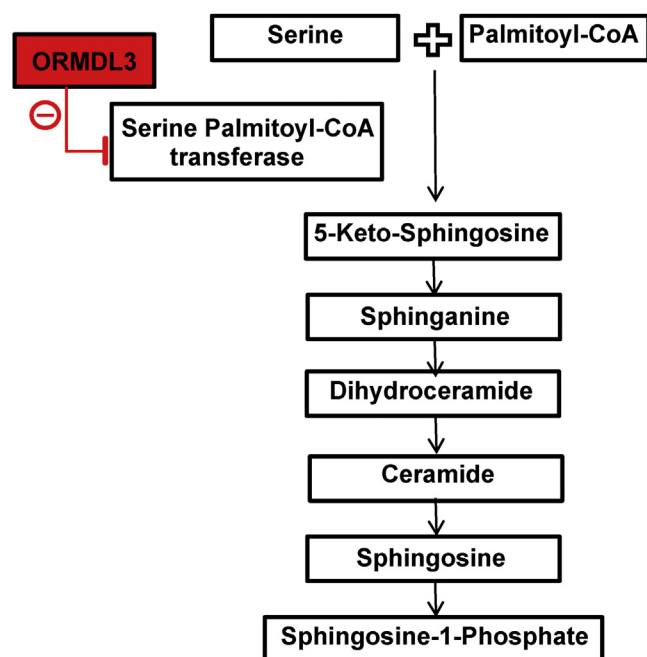


FIG E1. ORMDL3 inhibits the enzyme SPT, which regulates the first step of sphingolipid synthesis (conversion of serine and palmitoyl-coA to 5-keto-sphingosine) as described.^{E15} 5-Keto-sphingosine is metabolized through intermediary steps (sphinganine, dihydroceramide, ceramide, and sphingosine) to S1P.

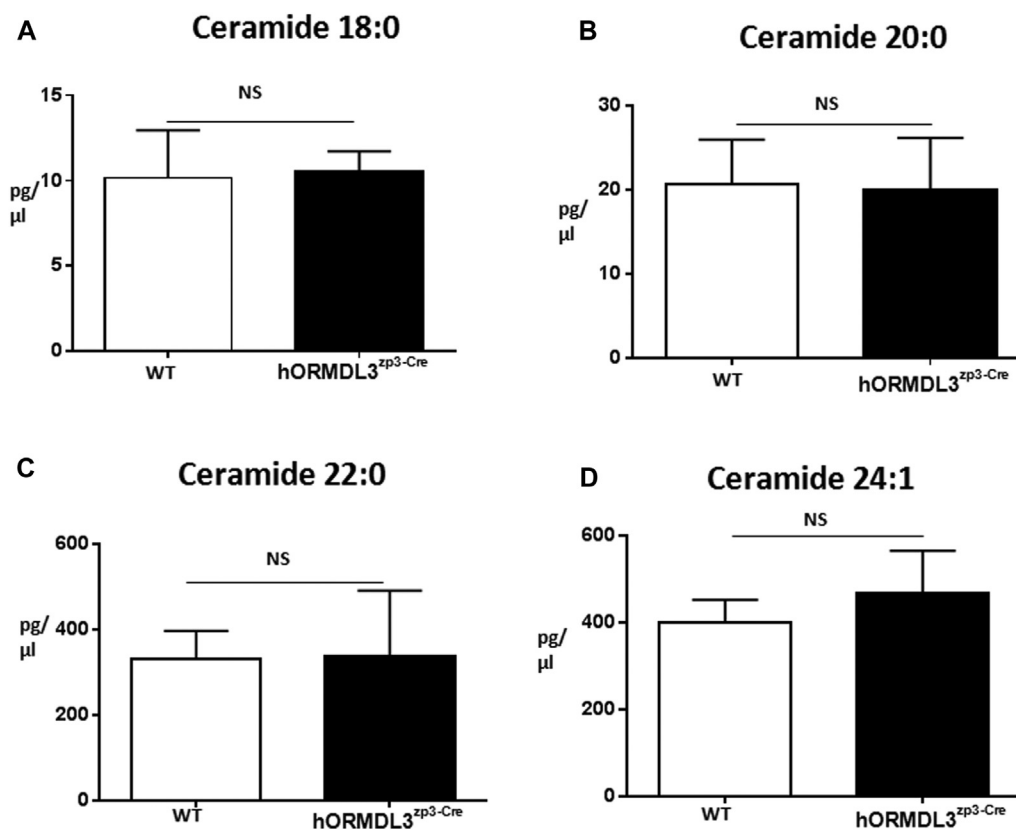


FIG E2. Levels of serum ceramide in hORMDL3^{zp3-Cre} mice. Levels of serum ceramides in hORMDL3^{zp3-Cre} mice and WT mice were quantitated by LC-MS/MS. Levels of only serum ceramide 24:0 ($P < .01$) (Fig 1) (but not ceramide 18:0 [A], ceramide 20:0 [B], ceramide 22:0 [C], and ceramide 24:1 [D]) (Fig E1) were decreased in serum in hORMDL3^{zp3-Cre} mice compared with WT control. LC-MS/MS, Liquid chromatography-tandem mass spectrometry.

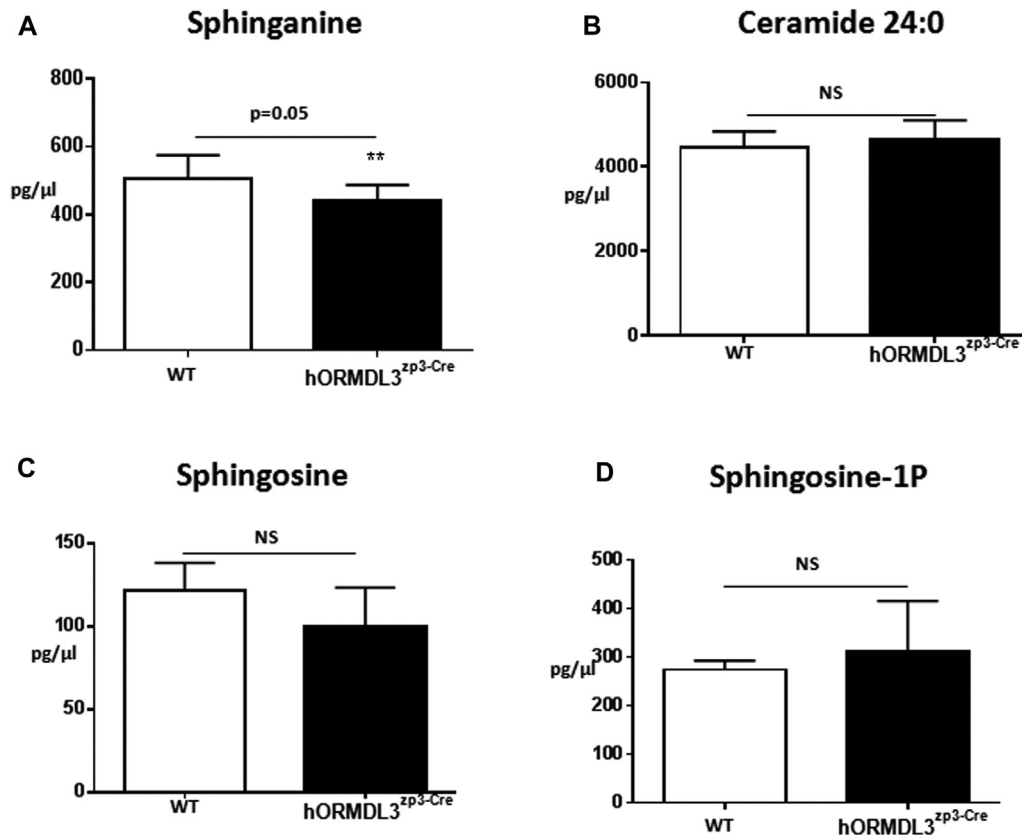


FIG E3. Levels of lung sphingolipids in hORMDL3^{zp3-Cre} mice. Levels of lung sphingolipids in hORMDL3^{zp3-Cre} mice and WT mice were quantitated by LC-MS/MS. hORMDL3^{zp3-Cre} mice had significantly reduced lung levels of sphinganine ($P < .05$) (A), but no reduction in ceramide 24:0 (B), sphingosine (C), or S1P (D) compared with WT control. LC-MS/MS, Liquid chromatography-tandem mass spectrometry.